NEURAL REGULATION OF CHROMATOPHORE FUNCTION IN CEPHALOPODS

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The long term goal of this research project was to elucidate some of the neural mechanisms underlying Body Pattering Behavior, the ability of cephalopod mollusks to generate numerous and highly complex body patterns. Cephalopods, which include octopus, squid and cuttlefish, are the only animals able to generate active body patterns directly controlled by the nervous system. These animals form numerous static and dynamic patterns used for a variety of purposes, including camouflage and inter- and intra-specific communication.

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FROM: Nathan Tublitz
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SUMMARY
The long term goal of this research project was to elucidate some of the neural mechanisms underlying **Body Pattering Behavior,** the ability of cephalopod mollusks to generate numerous and highly complex body patterns. Cephalopods, which include octopus, squid and cuttlefish, are the only animals able to generate active body patterns directly controlled by the nervous system. These animals form numerous static and dynamic patterns used for a variety of purposes, including camouflage and inter- and intra-specific communication. The patterns made by these animals are unique because of their rich detail, speed of formation (within 500 ms), and high degree of plasticity. We have been studying Body Patterning Behavior in the European cuttlefish *Sepia officinalis*, which arguably generates the most detailed and varied body coloration patterns in the animal kingdom. The focus of this research is to understand the neural control of the color producing elements, the chromatophores. The specific objectives of this project address several key issues of chromatophore regulation.

OBJECTIVES
The four specific objectives/aims of this project were:

1. Identify the neurotransmitters controlling yellow and reddish-orange chromatophores;
2. Clarify the role of the FMRFamide-related peptides (FaRPs) in chromatophore function;
3. Characterize the central location and properties of the chromatophore motoneurons; and,
4. Determine the rules underlying addition of new chromatophores during maturation and growth.

SUMMARY OF FINDINGS:

Specific Aim #1: We determined that the yellow and reddish-orange chromatophores are controlled by three transmitters, glutamate, the FaRPs, and serotonin. Glutamate and the FaRPs serve as the fast and slow excitatory transmitters, respectively, in this system whereas serotonin acts as the inhibitory transmitter.

Specific Aim #2: We isolated, cloned and sequenced two new FaRP genes in cuttlefish that code for 8 novel neuropeptides, determined their expression pattern in the cuttlefish CNS, and demonstrated that these peptides each act to cause a slow excitation of the chromatophore muscles.

Specific Aim #3: We identified the location and properties of chromatophore motoneurons that activate chromatophores covering leucophores.

Specific Aim #4: We ascertained the rules by which new chromatophores are added to the existing chromatophore array during maturation and growth.

In addition,

1) We determined that there are only two color types of chromatophores in cuttlefish, not three as previously assumed.

2) We quantitatively assessed the rate of regeneration of a severed arm in two species of cuttlefish.

3) We identified, cloned and sequenced a specific Hox gene, POST2, which has an alternative transcript only expressed in regenerating arm tissue.

4) We quantitatively measured the rate of regeneration of a severed arm in a related cephalopod species, *Octopus vulgaris* with the Fiorito lab at the Stazione Zoologica in Napoli, Italy and showed that regeneration follows a different time course than that in cuttlefish.

5) We performed a transcriptomal analysis on 6 different cuttlefish tissues, including the brain, retina, chromatophore layer, normal arm and arms 24 and 48 hrs after transection.

6) We generated an *Octopus vulgaris* genome survey to determine the size of the Octopus genome in collaboration with the Fiorito lab at the Stazione Zoologica in Napoli, Italy.

7) We began a large scale effort in collaboration with the Human Genome Sequencing Center at the Baylor College of Medicine to fully sequence, assemble and annotate the cuttlefish genome, which is 50% larger than the human genome. To date we have completed the sequence and are in the process of assembly and annotation.

DETAILS OF FINDINGS:
Specific Aim #1: Identify the neurotransmitters controlling yellow and reddish-orange chromatophores. The goal of this aim was to identify the neurotransmitters involved in the regulation of the three color types of chromatophores: black, reddish-orange and yellow. As

information exists only for the neuroregulation of black chromatophores, the focus was on the other two color types. During this grant period we assessed the effects of specific transmitter candidates on yellow chromatophores using a novel, real-time, digital data collection procedure to more precisely and rapidly analyze the effects of various transmitters on chromatophore activity. We did not study the reddish-orange chromatophores for reasons provided at the end of this section.

The real-time digital data collection system is a significant advance in analyzing chromatophore activity. Previously data collection was limited to procedures with slow response times. The new system enabled us to gather chromatophore response data, *i.e.,* changes in chromatophore area, in the 10 millisecond accuracy range, two orders of magnitude faster than any other known procedure. These measurements employed RGB threshold and polygon masking scripts to isolate single chromatophores of interest and to convert them into binary images for area computation. Using this system, we began to assess the effects of specific transmitter candidates on yellow chromatophores.

Glutamate caused a dose-dependent increase in expansion when applied to yellow chromatophores (Figure 1; upper left panel). Individual chromatophores responded to glutamate rapidly, within 15 sec of transmitter application (Figure 1, upper right panel). The diameter of individual yellow chromatophores increased on average approximately 10 fold in response to glutamate (Figure 1, lower panels).

Yellow chromatophores also expanded in response to application of the tetrapeptide FMRFamide in a dose-dependent manner (Figure 2, upper left panel). It is important to note that the onset and offset of the FMRFamide-dependent chromatophore response were significantly slower than that for glutamate (Figure 2, bottom left panel). We also tested serotonin on yellow chromatophores and found that serotonin caused chromatophore condensation in a dose-dependent fashion (Figure 2, right panels). Taken together, these data show that yellow chromatophores receive three qualitatively different input signals: fast excitatory (glutamate), slow excitatory (FMRFamide) and inhibitory (serotonin).

We also used the real-time digital data acquisition system to more accurately analyze the effects of glutamate, FMRFamide and serotonin on black chromatophores. Figure 3 shows the results of these experiments. Glutamate (Figure 3, top two panels) and FMRFamide (middle two panels) caused fast and slow chromatophore expansion, respectively. In contrast, serotonin produced chromatophore condensation (Figure 3, bottom two panels). These results are very similar to those observed in the yellow chromatophores (Figures 1 & 2).

We started to repeat these experiments on the reddish-brown chromatophores however we had serious difficulty identifying them *in vivo*. Direct images of skin samples (Figure 1, bottom panels) using high quality imaging equipment failed to find red-brown chromatophores, which raised the question of whether they exist. Additional experiments, reported elsewhere in this report, suggest that cuttlefish only have two types of chromatophores, brown-black and yellow (see below for details).

Specific Aim #2: Clarify the role of the FMRFamide-related peptides (FaRPs) in chromatophore function. During this grant period we isolated, identified and sequenced a cDNA

from a novel gene in *Sepia officinalis* coding for 4 new FaRPs. This gene was named SOFaRP$_2$ (GenBank ID: GU388435) because it was the second FMRFamide-Related Peptide (FaRP) gene identified in the cuttlefish *Sepia officinalis* (SO).

The SOFaRP$_2$ cDNA sequence is 835 bp in length, with an open reading frame of 567 bp encoding 188 amino acids (Figure 4). The start codon ATG is located at nucleotides 97-99 and the stop codon TAA is located at nucleotides 661-663. There are 96 base pairs of untranslated region (UTR) at the 5' end. A stop codon TAG in frame with the encoded protein is located at nucleotides 46-48. The sequence flanking the start codon is AATATGG, with the third nucleotide upstream of the start codon being A and the first nucleotide downstream of the start codon being G. These two nucleotides together suggest that this flanking region is a strong Kozak consensus sequence. The 3' UTR of 172 bp includes three AWTAAA polyadenylation signal site (where W stands for A or T) for the initiation of polyadenylation at 3' end mRNA.

The open reading frame of the SOFaRP$_2$ cDNA encodes a predicted protein of 188 amino acid residues including 4 different FaRPs, one copy of NSLFRF, three copies of GNLFRF, one copy of TIFRF and one copy of PHTPFRF. Each FaRP ends with RF and is likely post-translationally amidated at the C-terminus given the presence of a glycine immediately following the RF residues. Each FaRP is flanked by basic residues Lysine-Arginine at the amino terminus and Lysine-Arginine or Arginine at the carboxyl terminus, which serve as internal proteolytic cleavage sites during post-translational processing.

A BLAST search was performed to find homologous nucleotide sequences in the Reference mRNA Sequences databases and no significant similarity was found.

To examine the expression of SOFaRP$_2$ gene in the *Sepia officinalis* brain, *in situ* hybridization was performed using an antisense riboprobe to the full-length SOFaRP$_2$ mRNA. Specific staining was observed in multiple regions of the cuttlefish brain (Figure 5). Our results focused on the regions of the brain involved in the generation of body patterns. No staining was detected using the control sense riboprobe to SOFaRP$_2$ mRNA (Figure 5A).

Positive staining was observed in the posterior suboesophageal mass in several locations including the posterior chromatophore lobes (PCL) and fin lobes (Figure 5B&C). About 40% of the neurons were stained in posterior chromatophore lobe (PCL). The staining was mostly concentrated in the somata (Figure 5B). The fin lobes were labeled less intensively, with about 20% of the neurons positively stained (Figure 5C).

In the middle suboesophageal mass, only the anterior chromatophore lobes (ACL) were clearly labeled. Staining was localized to the front of the anterior pedal lobe and in the dorsal part of the ACL (Figure 5D). The supraoesophageal mass also showed positive staining in multiple lobes including the lateral basal lobes, which exhibited SOFaRP$_2$ expression in nearly 50% of its somata. (Figure 5E). The optic lobes also displayed positive labeling by the SOFaRP$_2$ probe (Figure 5F).

SOFaRP$_2$ gene expression was also found in other brain regions including the brachial lobes, subvertical lobe, superior frontal lobe and dorsal basal lobe (data not shown), areas involved in feeding, learning and memory formation.

To determine possible roles of SOFaRP$_2$-encoded neuropeptides on black chromatophores, each of the four putative neuropeptides encoded in the SOFaRP$_2$ gene was tested individually using the *in vitro* chromatophore bioassay described earlier in this report. Three of the four putative peptides,

GNLFRFamide, PHTPFRFamide and NSLFRFamide, each caused significant chromatophore expansion within 50 sec of peptide application when applied at a concentration of 10^{-5} mol l^{-1} (Figure 6A-C). Each peptide caused an approximate 10 fold increase in the area of all tested chromatophores (Figure 6A-C) and these increases returned to basal levels usually after the peptide application was washed out with artificial sea water. In contrast, TIFRFamide at a concentration of 10^{-5} mol l^{-1} did not induce any significant changes in the size of black chromatophores (Figure 6D).

Specific Aim #3: Characterize the central location and properties of the chromatophore motoneurons. This aim focused on understanding the electrophysiological properties and location of the motoneurons controlling the chromatophore muscles. A post-doc in the lab identified various locations in the brain that activate chromatophore muscles when stimulated. An interesting, new observation was the presence of specific chromatophore motoneurons which only activate chromatophores located directly dorsal (i.e., above) to the underlying leucocytes (Figure 7, left panel). This finding indicates an unusual organizational pattern of a subset of chromatophore motoneurons and suggests the importance of hiding leucocytes during body patterning displays. A second, interesting finding was the discovery of complex patterns when cuttlefish were immersed in 10^{-3}M glutamate. As partially shown in the still image in Figure 7 (right panel), glutamate exposure produced complicated body waves similar to those seen in nature. This observation provides the foundation for future experiments on central control of multiple chromatophores.

Specific Aim #4: Determine the rules underlying addition of new chromatophores during maturation and growth. During this grant period we completed the aim of identifying the principles by which new chromatophores are inserted into the existing chromatophore array during normal growth and during regeneration.

In a set of regeneration experiments, we found that chromatophores return to the skin in a precise sequence: the first chromatophores to appear in the newly regenerating skin tissue are inserted much further apart from each other than chromatophore spacing in control tissue (Figure 8). The distance between new and existing chromatophores was determined using a quantitative microscopic measurements to determine the separation between the two chromatophores, a measurement we called interchromatophore diameter (ICD). Subsequent new chromatophores fill in the gaps until the skin has grown back completely and the spacing of new chromatophores is identical to that in untreated control tissue.

To determine if the rules for new chromatophore insertion during regeneration also pertain to normal development, measurements of ICDs of newly added fin chromatophores were collected during normal growth and maturation and compared to ICDs from existing (control) chromatophores (Figure 9). Data for each chromatophore type (yellow, reddish-brown and black) were obtained from seven juvenile cuttlefish and analyzed separately. No new chromatophore was used as a data point for ICD analysis until its color became unequivocally distinguish-able as either yellow, reddish-brown, or black.

As a general rule, approximately half of the new chromatophores were inserted along the outside edge of existing chromatophores and the remainder added to the interior in between pre-existing chromatophores. The overall mean ICD of newly inserted black chromatophores was 15 +/- 0.22 microns (SEM) over the 30 day measurement period (Figure 9a). In contrast, the overall mean ICD

for existing (control) black chromatophores was significantly larger at 19 +/- 0.036 microns (Figure 9a). Daily mean ICDs of new or existing chromatophores did not vary significantly during the data collection period.

The same general pattern was observed for the reddish-brown (Figure 9b) and yellow chromatophores (Figure 9c). New reddish-brown chromatophores were added at a mean ICD of 19 +/- 0.34 microns compared to their mean control ICD of 21 +/- 0.12 microns, and new yellow chromatophores appeared at a mean ICD of 11 +/- 0.23 microns compared to their control data of 15 +/- 0.046 microns. Statistical analysis using a one-way ANOVA found that existing (control) black, yellow and reddish-brown ICDs were significantly different from each other. Control versus new ICDs were also significantly different from each other for each chromatophore color type, with the existing ICDs being greater than their respective newly added ICD values. The ratios of the mean ICD of new and existing chromatophores for each color type revealed that new chromatophores were always inserted closer to their nearest neighbors than the normal chromatophore spatial frequency (0.74, 0.85, & 0.57 for black, reddish-brown and yellow chromatophores, respectively). These data also indicate a higher density (*i.e.,* smaller ICD) of yellow chromatophores in the fin than either black or reddish brown chromatophores (Figure 9).

To understand the maturation process of newly formed chromatophores, we individually tracked 516 chromatophores from their initial appearance to their adult phenotype. New chromatophores always appeared first as very small, pale orange cells, and were not classifiable as any of the three mature chromatophore colors (Figure 10). Following their initial appearance, all new chromatophores slowly darkened and, in the case of black chromatophores, grew larger over the course of 27-30 days until they were the same color and size as existing chromatophores of the same color type. For example, black chromatophores turned from an initial pale orange, to a reddish-orange color, then to dark brown, and finally to their mature dark brown-black color similar to that of surrounding black chromatophores (Figure 11). This darkening developed slowly over the course of a few weeks rather than in a step-wise manner in which color changed dramatically from day to day. During the time they were darkening, developing black chromatophores were never recognizable as either yellow or reddish-brown chromatophores. New reddish-brown and yellow chromatophores developed similarly to black chromatophores, starting out as small pale orange cells which changed color until they closely resembled surrounding chromatophores of the same color type. Once a chromatophore became recognizable as a fully grown black, reddish-brown, or yellow chromatophore, its color remained unaltered for the remainder of the observation period.

Our data show that chromatophore growth and development are highly organized and structured. From our observations, we propose 5 guiding principles of chromatophore growth and maturation:

> 1) Each of the three chromatophore cell types - black, reddish brown, and yellow - are present at different spatial frequencies in the cuttlefish fin;

> 2) During normal growth, new chromatophores are inserted at a higher spatial frequency than control chromatophores of the same color type;

> 3) In regenerating tissue, new black chromatophores are initially added at low spatial frequencies. As regeneration continues, new black chromatophores appear at increasing

spatial frequencies until they are inserted at a spatial frequency higher than observed in control tissue;

4) All chromatophores first appear as pale orange cells and slowly darken into their respective color types without passing through intermediate color stages; and,

5) New black chromatophores undergo a doubling in size as they mature while, reddish-brown and yellow chromatophores do not grow at all after they are inserted in the dermis.

Our data supports a new model of chromatophore development (Figure 12). Chromatophores started out as small, pale orange cells that developed directly into either yellow, reddish-brown, or black chromatophores without passing through either of the other two colors. We looked very closely but did not observe any reddish-brown chromatophores passing through a transient yellow phase or a mature black chromatophore which began yellow in color and went through a reddish-brown phase before becoming black. All yellow chromatophores observed on the first day of filming remained yellow at the end of the 30 day observation period, during which we observed the appearance of many new black chromatophores. These results suggest that chromatophores do not mature by going through all 3 colors as has been suggested in the literature.

In addition to the above results, we also worked on the following issues during this grant period:

1) We determined that there are only two color types of chromatophores in cuttlefish, not three as previously assumed. During this grant period we began to consider the possibility that perhaps the previous work on chromatophores in cuttlefish was incorrect and that there were only two color types of chromatophores (orange and black) in the cuttlefish not three (yellow, reddish-brown and black). We tested this hypothesis using several independent methods: 1) quantitative measurements of chromatophore color types in different regions of the cuttlefish body using naïve observers; 2) video analyses of chromatophore activity to assess the number of chromatophore layers in the skin; and, 3) histological investigations of the cuttlefish skin to determine the number of chromatophore layers and color types (Figure 13). The results from these investigations appear to confirm our initial hypothesis that the skin of the cuttlefish *Sepia officinalis* contains only two chromatophore color types, orange and black.

2) We quantitatively assessed the rate of regeneration of a severed arm in two species of cuttlefish. Based on our regeneration work on the cuttlefish skin (see Specific Aim #4 above), we were interested in determining whether the arms of cuttlefish were also capable of regeneration and if so, to what extent. To address this issue, individual arms were amputated from juvenile *Sepia officinalis* cuttlefish and their regrowth was monitored every second or third day. Arm regrowth progressed quickly and a fully functional, full length arm was observed at approximately day 39 after arm ablation (Figure 14A & B). This work was repeated in a second cuttlefish species (*Sepia pharaonis*) with similar results (Figure 14C & D).

For both species a regeneration time line was developed consisting of 5 post-amputation stages (Figure 15). The first post-amputation stage consists of a frayed leading edge. By Stage II, the frayed edge has healed and has become smooth and rounded. Stage III is heralded by a small yet unmistakable growth bud on one side of the leading edge which develops into a narrow, extending tip which announces the beginning of Stage IV. The final stage, Stage V, is identifiable by the

elongation of the tip. By the end of stage V, the regenerated arm is completely indistinguishable morphologically and functionally from the other arms. Growth rates of the regenerating arm in both species were measured and we found a high degree of variability depending on regeneration stage (data not shown). New suction cups were observed from Day 3 post—ablation and appeared to be always added at the distal tip of the re-growing arm (data not shown). New black and yellow chromatophores were observed at the severed edge within 24 hr of ablation and were continuously added throughout all regeneration stages (data not shown).

We also assessed the animals' behavior throughout the regeneration process (Figure 16). Several behaviors were impaired as a result of amputating a single arm. These including swimming, food manipulation, body postures and brown tip behavior in which the tips of the arm darken briefly as the animal settles onto the tank substrate. All behaviors eventually returned to normal however the time course of recovery from behavioral impairment varied with the specific behavior (Figure 16).

3) We identified, cloned and sequenced a specific Hox gene, POST2, which has an alternative transcript only expressed in regenerating arm tissue. During the grant period we also began to investigate the molecular mechanisms underpinning the process of arm regeneration in *Sepia officinalis*. The hypothesis we tested was that regeneration activated one of the 9 homeobox (Hox) genes found in cephalopods. Analyses of the expression of each of the 9 Hox genes revealed that only one, POST2, the Hox gene involved in the formation of the most posterior parts of the body during embryonic development, was expressed during regeneration (Figure 17A). What made this gene even more interesting was the existence of two POST2 transcripts, a shorter one that was expressed before amputation and a 60 bp longer transcript expressed after arm ablation (Figure 17B).

4) We quantitatively measured the rate of regeneration of a severed arm in a related cephalopod species, *Octopus vulgaris* with the Fiorito lab at the Stazione Zoologica in Napoli, Italy and showed that regeneration follows a different time course than that in cuttlefish. We followed up on our studies on arm regeneration in cuttlefish by conducting a series of experiments in the octopus *O. vulgaris*, which also has been shown to regenerate its arms after ablation. One arm from each animal was ablated to 20% of its original length. The length of the experimental arm, the contralateral control arm, and the remaining 6 untreated arms were measured one week after ablation and every two weeks thereafter. The results, shown in Figure 18, indicate that Octopus vulgaris juveniles are capable of significant regeneration however none of the regenerating arms ever reached the length of the control arms unlike in cuttlefish. These experiments were carried out in the Fiorito laboratory at the Stazione Zoologica in Napoli, Italy.

5) We performed a transcriptomal analysis on 6 different cuttlefish tissues, including the brain, retina, chromatophore layer, normal arm and arms 24 and 48 hrs after transection. For each tissue, RNA was isolated, sequenced using alumina sequencing techniques and assembled with the Velvet de novo assembly program. Initial annotation was performed using several diverse annotation programs. Our initial analyses included identification of the top matches for known proteins (Figure 19), molecular function (Figure 20) and cellular location (Figure 21).

6) We generated an *Octopus vulgaris* genome survey to determine the size of the Octopus genome in collaboration with the Fiorito lab at the Stazione Zoologica in Napoli, Italy.

Estimates of cephalopod genome size ranges from ~1 Gb (Nautilus) to over 5 Gb (Octopus). Previous work on the size of the *Octopus vulgaris* genome varies from 2.5 Gb to 5.5 Gb. In collaboration with the Fiorito lab in Napoli, Italy, we decided to re-evaluate the genome size of *O. vulgaris* using modern techniques. The results from KMER analyses resulting in a genome of ~2.5 Gb (Figure 22). However this method does not account for gene duplications and heterozygosities. An independent method that uses 4 different sized insert libraries and a nearly 50X genome coverage and which accounts for high proportion of gene duplications and heterozygosities produced a more realistic figure of 5.0 Gb (Figure 23). If correct, then the octopus genome is surprisingly 60% larger than the human genome.

7) We began a large scale effort in collaboration with the Human Genome Sequencing Center at the Baylor College of Medicine to fully sequence, assemble and annotate the cuttlefish genome, which is 50% larger than the human genome. To date we have completed the sequence and are in the process of assembly and annotation. Using sequencing libraries with insert sizes of 180bp, 500bp and 3kb, we assembled the cuttlefish genome using a three step procedure. First, an initial assembly was produced using ALLPATHS-LG. Next, additional scaffolding was performed using ATLAS-Link and finally the contigs were extended and gaps filled using ATLAS-GapFill.

This three-step process resulted in an initial draft assembly with the following statistics:

Total contig length: 3,279,735,617 bases
Total number of contigs: 2,058,491
Contig N50 length: 2,774 bp
Number of contigs >10 kb: 27,392
Scaffold N50 length: 225.7 kb

Preliminary annotation data uncovered thousands of genes with known homologues in other non-cephalopod species (Figure 24).

RESEARCH PUBLICATIONS FUNDED BY THIS GRANT:

Yacob, J., A.C. Lewis, A. Gosling, D. H.J.St Hilaire, L. Tesar, M. McRae and N.J. Tublitz (2011) Principles underlying addition of new chromatophores in the European cuttlefish Sepia officinalis. *J. Exp. Biol.,* **214**: 3423-3432.

Zhang, Z., E. Goodwin, P.K. Loi and N.J. Tublitz (2012) Molecular analysis of a novel FMRFamide-related peptide gene (SOFaRP2) and its expression pattern in the brain of the European cuttlefish *Sepia officinalis. Peptides* **34**: 114-119.

Goodwin, E., and N.J. Tublitz (2013) Video analyses of chromatophore activity in the European cuttlefish *Sepia officinalis. J. Exp. Marine Biol. & Ecol.*, **447**:156-159.

Zhang, Z, and N.J. Tublitz (2013) Expression of the SOFaRP$_2$ gene in the central nervous system of the European cuttlefish *Sepia officinalis. Neuropeptides* **47**: 147-155.

Tressler, J., F. Maddox, E. Goodwin, Z. Zhang and N.J. Tublitz (2014) Arm regeneration in two species of cuttlefish, *Sepia officinalis* and Sepia *pharaonis*. *Invert. Neurosci.,* **14: 37-49. DOI 10.1007/s10158-013-0159-8.**

Fiorito, G., A. Affuso, D. B. Anderson, J. Basil, L. Bonnaud, G. Botta, A. Cole, L. D'Angelo, P. De Girolamo, N. Dennison, L. Dickel, A. Di Cosmo, C. Di Cristo, C. Gestal, R. Fonseca, F. Grasso, T. Kristiansen, M. Kuba, F. Maffucci1, A. Manciocco, F. C. Mark, D. Melillo, D. Osorio, A. Palumbo, K. Perkins, G. Ponte, M. Raspa, N. Shashar, J. Smith, D. Smith, A. Sykes, R. V. López, N. Tublitz, L. Zullo, and P. Andrews (2014). Cephalopods in Neuroscience: Regulations, Research and the 3Rs. *Invert. Neurosci.,* **14:** 13-36. **DOI 10.1007/s10158-013-0165-x**

Zhang, Z. and N.J. Tublitz (2015) Identification and expression of a regeneration-specific, splice variant of the POST Hox gene in arm regeneration in the European cuttlefish *Sepia officinalis.* In preparation.

Zarrella, I., G. Fiorito and N J. Tublitz (2015) Morphometric and behavioral analyses of arm regeneration in the common octopus *Octopus vulgaris*. In preparation.

INVITED RESEARCH TALKS DURING THE GRANT PERIOD (06/2009-02/2015)

University of Pisa, Italy
University of Naples, Italy
Oregon Summer Science Experience, Corvallis OR
Institute of Decision Science, University of Oregon
Natural and Extremophilic Materials and Systems, Washington DC (4)
EuroCeph2011, Vico Equense, Italy
University of North Texas, Denton TX
Washington State University, Pullman WA
Stazione Zoologica Anton Dohrn, Naples, Italy (2)
Conference on Welfare of Cephalopods in Research, Vico Equense, Italy
Meeting of a European Consortium to sequence the *O. vulgaris* genome, Vico Equense, Italy
Conference on Sepia Breeding: Improving Reproduction Performance Based on Biological Knowledge, Centro di Ciencias do Mar (CCMAR), Faro, Portugal
Lawrence University, Appleton WI
Educational Testing Service, Princeton NJ
Le Museum Nationale d"Histoire Naturelle-Universite de Paris, France
Symposium on Advances in Invertebrate Neurobiology, Polish Neuroscience Society, Poznan, Poland
Symposium on Cephalopod Regeneration, Stazione Zoologica, Naples, Italy
Reed College, Portland OR
Davidson College, NC
St. Mary's College of Maryland
Cambridge University
University of Newcastle
University College, London

COLLABORATIONS
Dr. Roger Hanlon, Marine Biological Laboratories, Woods Hole, MA USA;
Dr. Graziano Fiorito, Stazione Zoologica, Naples, Italy
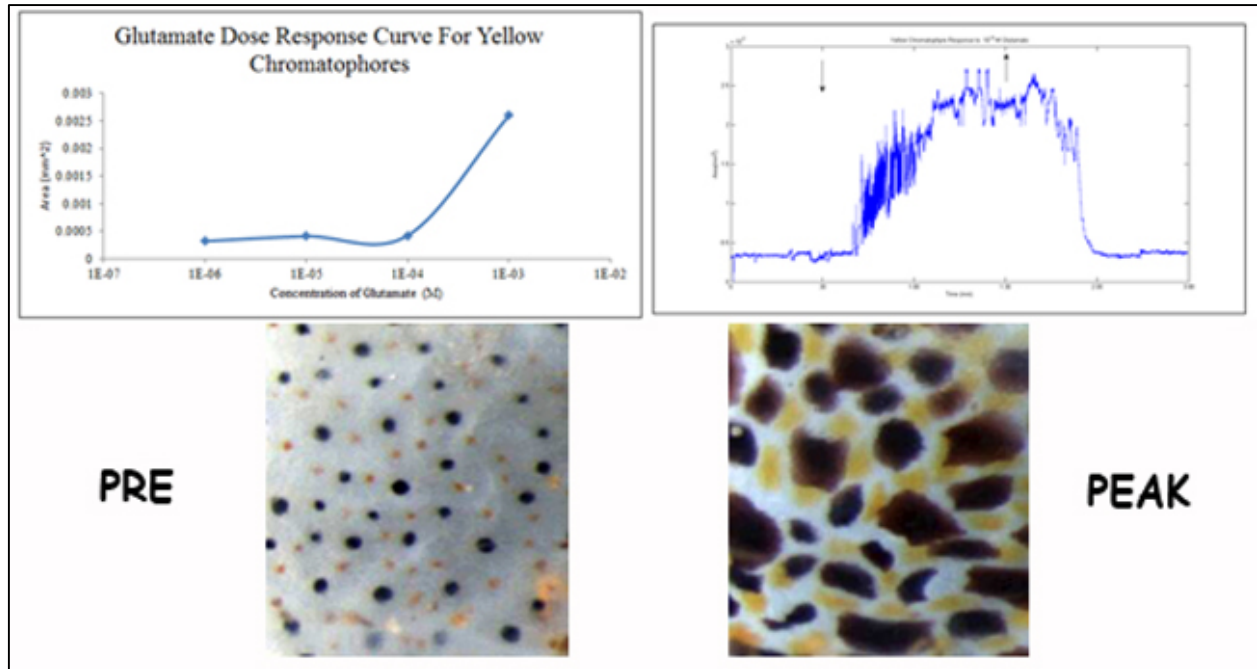
FIGURES

Figure 1. Effect of Glutamate on Yellow Chromatophores. Upper left: Glutamate dose response curve. Each point represents the mean +/- SEM (n=10). Upper right: Response of an individual yellow chromatophore to 10^{-3}M glutamate. Upwards = expanded. Up and down arrows represent glutamate application and washout, respectively. Lower panels: patch of skin with chromatophores before (PRE) & during (PEAK) glutamate application.
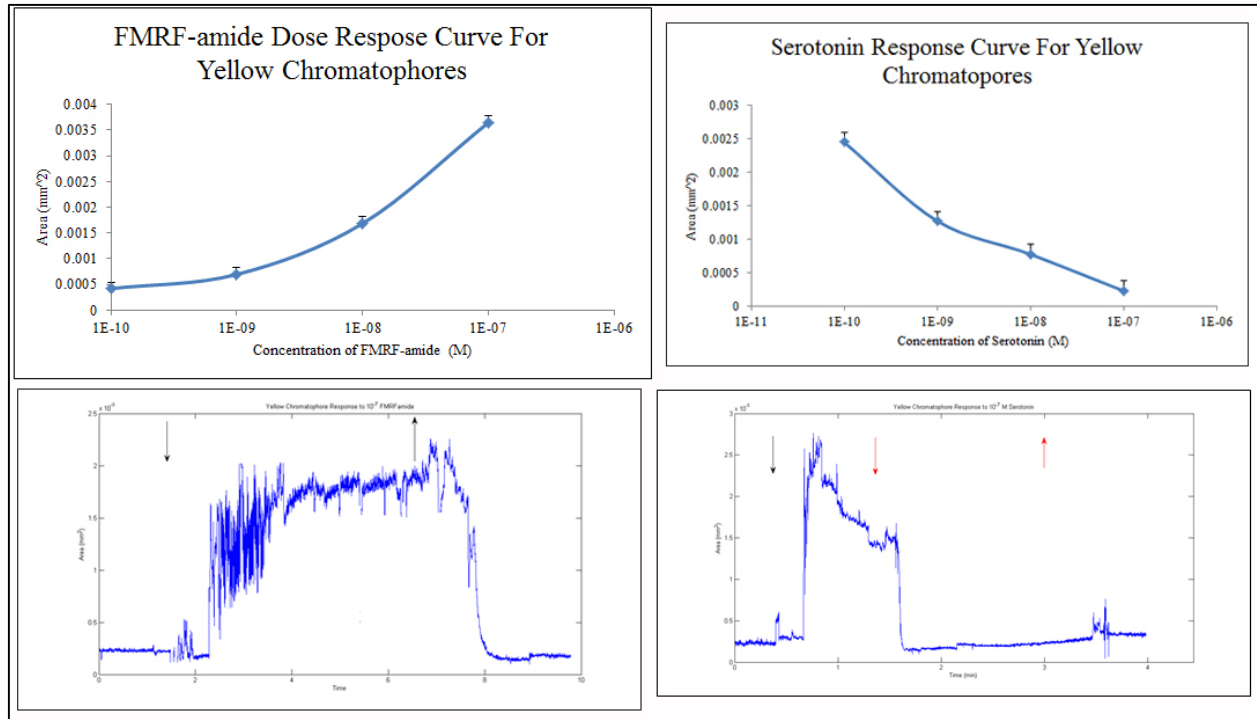
Figure 2. The Effects of FMRFamide and Serotonin (5-HT) on Yellow Chromatophores. Left panels: FMRFamide dose-response curve (upper) and individual chromatophore response to 10^{-7}M FMRFamide (lower). Right panels: Serotonin dose-response curve (upper) and individual chromatophore response to 10^{-7}M serotonin (lower). Each point in the upper panels represents the mean +/- SEM (n=10). Down arrows represent transmitter application (left panel, FMRFamide; right panel, glutamate followed by serotonin (red arrow)). Up arrows represent washout.
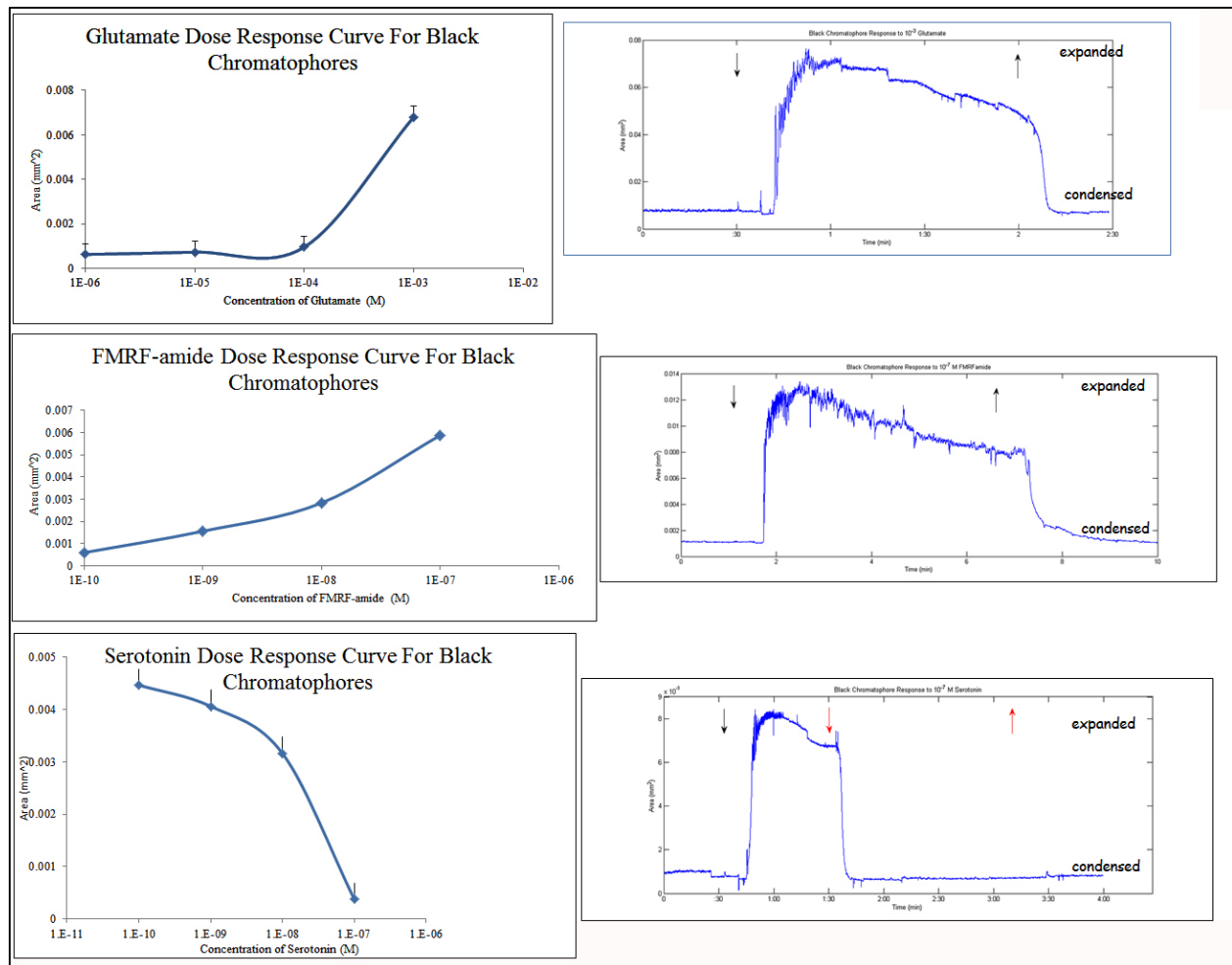
Figure 3. The Effects of Glutamate (top panels), FMRFamide (middle panels) and Serotonin (bottom panels) on Black Chromatophores. Concentrations and symbols as described in Figures 1 and 2.

```
  1   GCACGAGCCGAATCGCGTTTCTCGTACAGAATACAAAGCATTTCTTAGAAC
 52   CTGGGCTTAACAAATACAGCGAACCGGCCAACGGTCAGATCAAAT

 97   ATG GAA ACA AAA GTA ATG AGT TTG TTG GCG ACT GTG CTA
       M   E   T   K   V   M   S   L   L   A   T   V   L

136   ACC GTG TTC ATT GTG CAG ATA AAT TGC GAA GAC CTA CAC
       T   V   F   I   V   Q   I   N   C   E   D   L   H

175   AAA ATA CAA ACA GAC ACT TCC GGC ATT TCT AAT TTT ATC
       K   I   Q   T   D   T   S   G   I   S   N   F   I

214   GGT CTA CCA GAT GGA GAA GAA GGC GAA CTA GTG AGA TCC
       G   L   P   D   G   E   E   G   E   L   V   R   S

253   CCG ATT GTT GAC GAA TCA GCG CTC GGC ATT GAC GAT GTC
       P   I   V   D   E   S   A   L   G   I   D   D   V

292   GAC AAA CGC AAC AGC CTC TTC CGA TTC GGC AAG CGT GGA
       D   K   R   N   S   L   F   R   F   G   K   R   G

331   AAC CTC TTC CGG TTT GGT AAA CGC GGA AAC CTC TTC CGG
       N   L   F   R   F   G   K   R   G   N   L   F   R

370   TTT GGT AAA CGT GGA AAC CTC TTC CGT TTT GGA CGC GGT
       F   G   K   R   G   N   L   F   R   F   G   R   G

409   GGC AAC AAG GAC GAC CCC GAA AAC GAA GGA CTC AAA CGC
       G   N   K   D   D   P   E   N   E   G   L   K   R

448   ACC ATC TTT AGA TTC GGT AAA AGA GAC GGA CTC GAA GAC
       T   I   F   R   F   G   K   R   D   G   L   E   D

487   CTT TAC GAC TAC GAG GAT CCC TCA GTA CAA CAG GTG GCA
       L   Y   D   Y   E   D   P   S   V   Q   Q   V   A

526   CCA ACA GCT GGA GAC AAG CGC GGA TCA TTC TTC CGG TAT
       P   T   A   G   D   K   R   G   S   F   F   R   Y

565   GGA CGA AGC CGC ACT TTC TTT CGA TAC GGT AGA AGC ACT
       G   R   S   R   T   F   F   R   Y   G   R   S   T

604   GAT AAG AAC GCC GAG AAG AGG CCA CAC ACA CCC TTC CGA
       D   K   N   A   E   K   R   P   H   T   P   F   R

643   TTT GGA AGA GAA GAA GAG TAA
       F   G   R   E   E   E  STOP
```

Figure 4. Sequence of the open reading frame from a novel FaRP gene in the European cuttlefish *Sepia officinalis* involved in chromatophore regulation. The open reading frame codes for 4 neuropeptides (in red), each of which are amidated at the C-terminus using glycine residues (in green). The gene contains 3 copies of GNLFRFamide, and one copy each of NSLFRFamide, TIFRFamide **and** PHTPFRFamide. Furin excision sites (KR and R) are shown in purple.
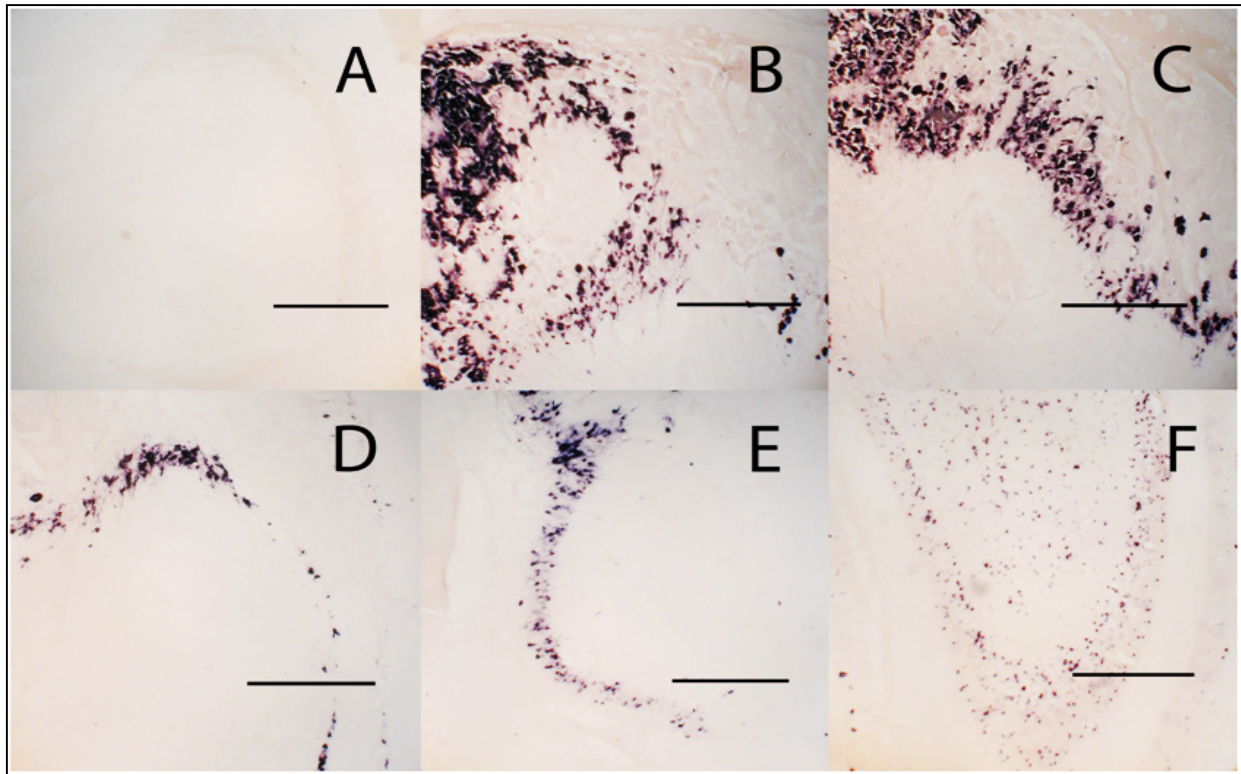
Figure 5. *In situ* hybridization with a full length SOFaRP$_2$ riboprobe showing that the SOFaRP$_2$ gene is expressed in the cuttlefish brain in regions implicated in chromatophore regulation. A: Medial section of the inferior frontal lobe hybridized with antisense riboprobe. B: Lateral sagittal section of the posterior chromatophore lobe. C: Lateral sagittal section of the fin lobe. D: Lateral sagittal section of the anterior chromatophore lobe. E: Lateral sagittal section of the lateral basal lobe. F: Tangential section of the optical lobe. Scale bars: 500 µm.
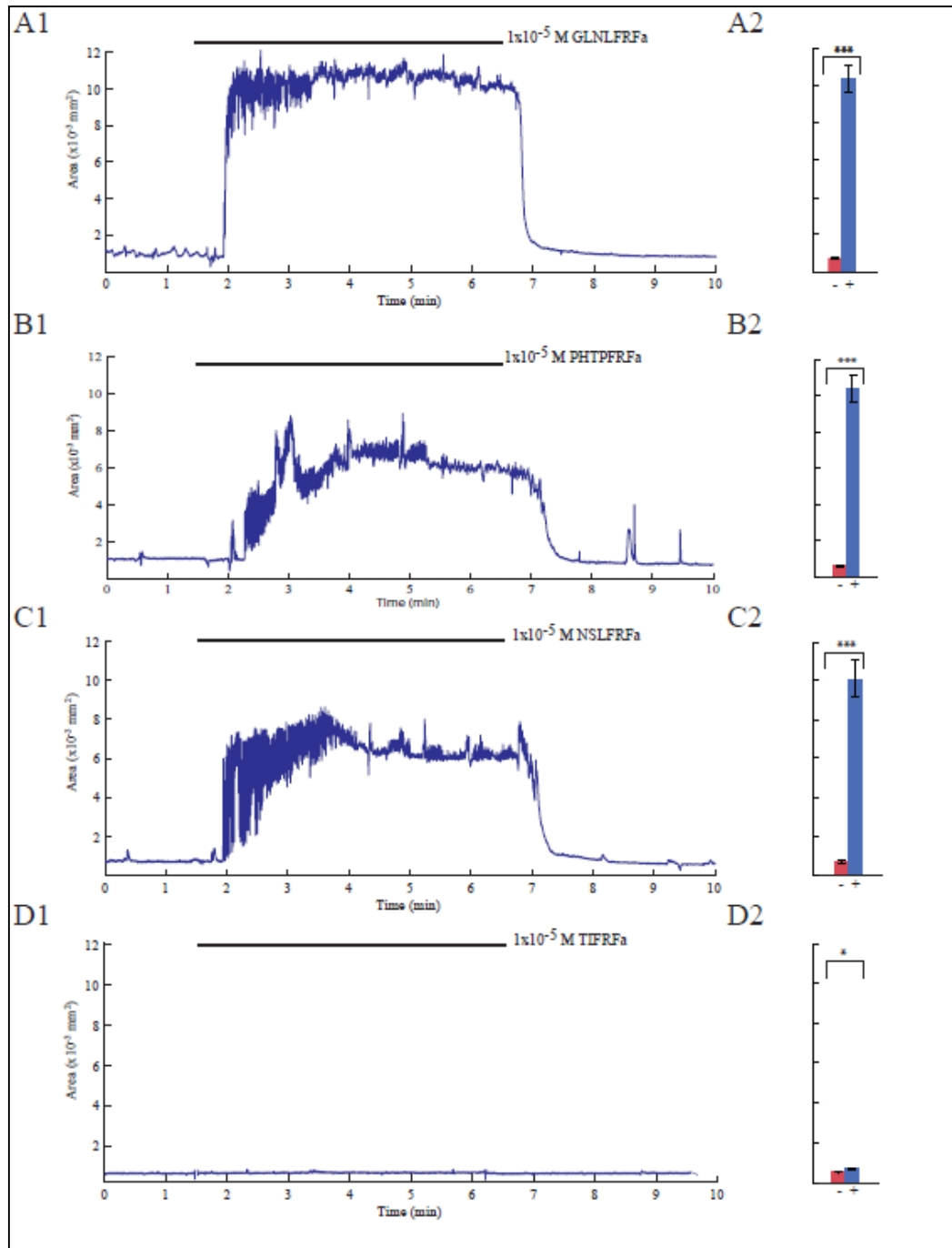
Figure 6. The effects of SOFaRP₂ peptides on black chromatophore activity in the European cuttlefish _Sepia officinalis_. A-D1: Changes in the area of individual black chromatophores in response to application of SOFaRP₂ peptides. Chromatophores were exposed to artificial sea water (ASW) for 1.5 min, then 5 min in ASW containing one of the FaRPs, and then washed in ASW for at least 3.5 min until the chromatophore area returned to basal levels. Black bar indicates period of peptide application. A-D2: Mean change in the area of black chromatophores 10 sec before (-, red bars) and maximum response after (+, blue bars) application of SOFaRP₂ peptides. Each histogram represents the mean area in mm² +/- SEM (n=15 for each histogram; ***, p<0.001; *, p< 0.01.)
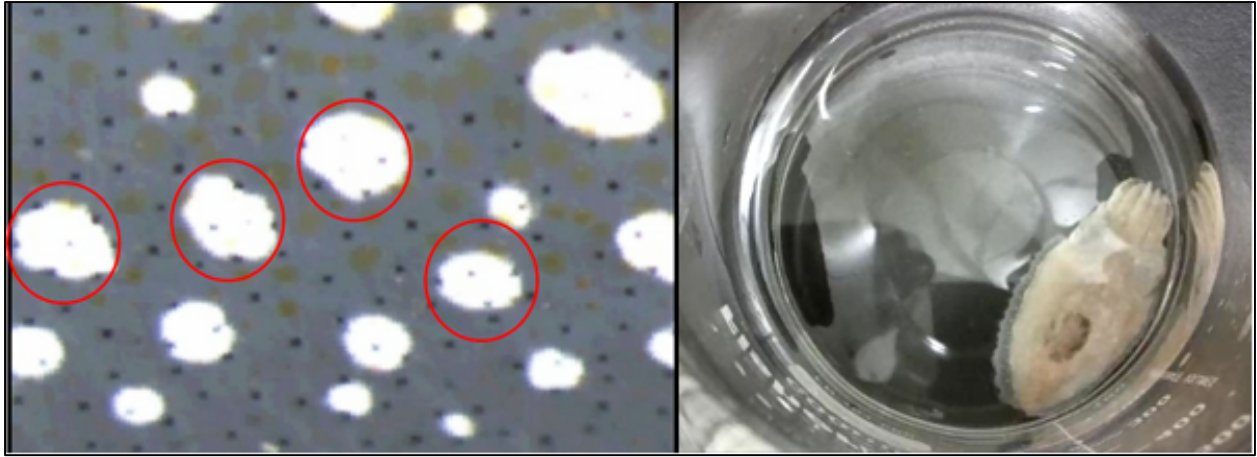
Figure 7. Functional organization of chromatophore motoneurons. Left panel, stimulation of a single brain chromatophore motoneuron only activates black chromatophores on top of leucocytes in the red circles. Right panel, image from a video showing glutamate induced chromatophore waves.
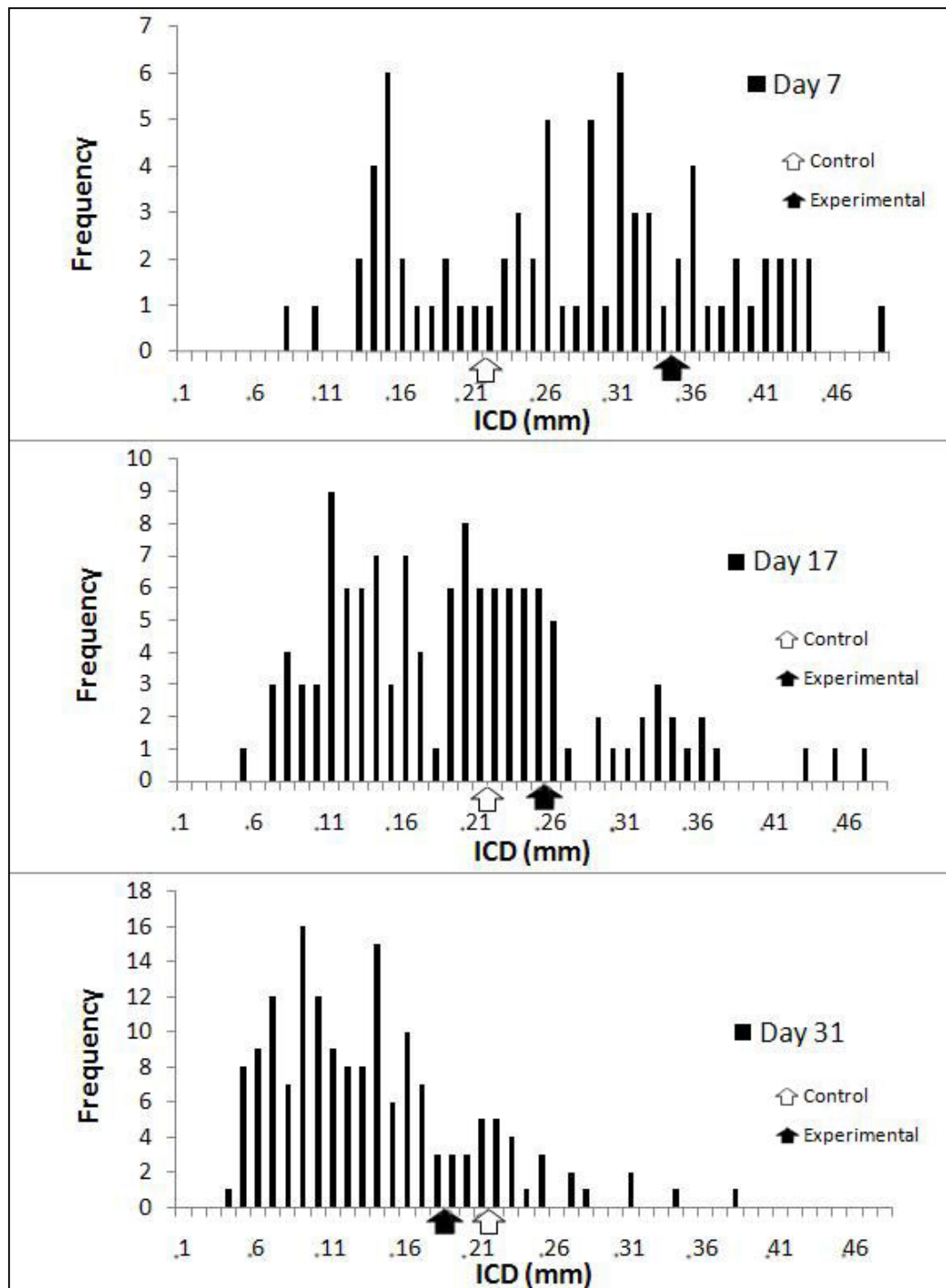
Figure 8. **Frequency histogram of inter-chromatophore distance (ICD) measurements from newly added black chromatophores during regeneration in juvenile European cuttlefish *Sepia officinalis*.** Days 7, 17 and 31 refer to days after excision of a patch of skin. Black and white arrows indicate the mean ICD from new and existing chromatophores, respectively, for each day.
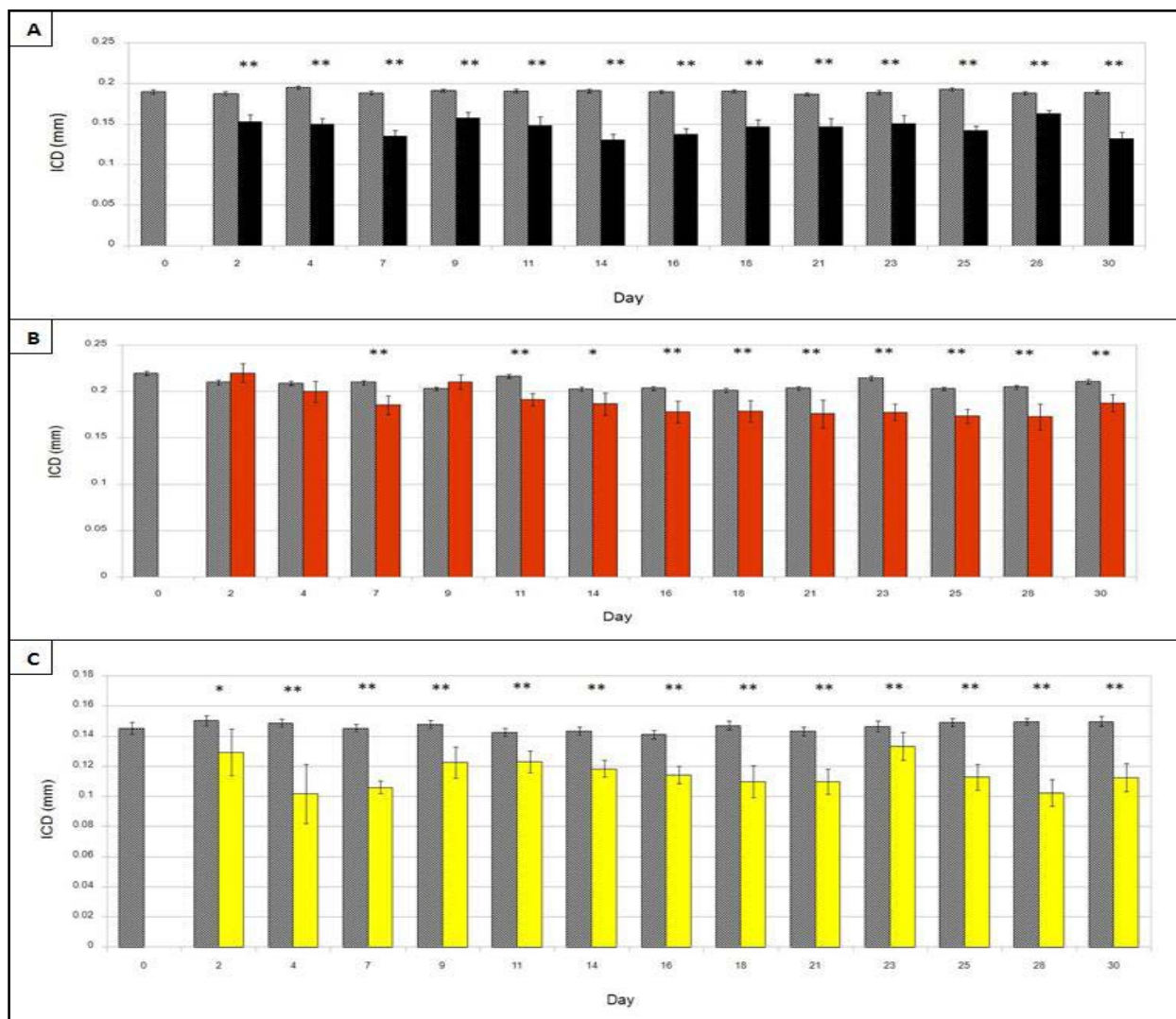
Figure 9. Mean daily inter-chromatophore distance (ICD) of black (top panel), reddish-brown (middle panel) and yellow (bottom panel) chromatophores from the European cuttlefish *Sepia officinalis* during normal growth. Mean ICDs (mm) from newly added (solid bars) or existing (hatched bars) chromatophores were obtained every second or third day for a 30 day period. Each solid bar represents the mean ICD from between 8 to 30 new chromatophores and each hatched bar represents the mean ICD from 100 existing chromatophores. Error bars represent +/- 1 SEM. A single or double asterisk (*, **) indicates a statistically significant difference between the mean daily ICD of newly added and existing chromatophores for that day at the $P < 0.05$ or $P < 0.01$ level, respectively.
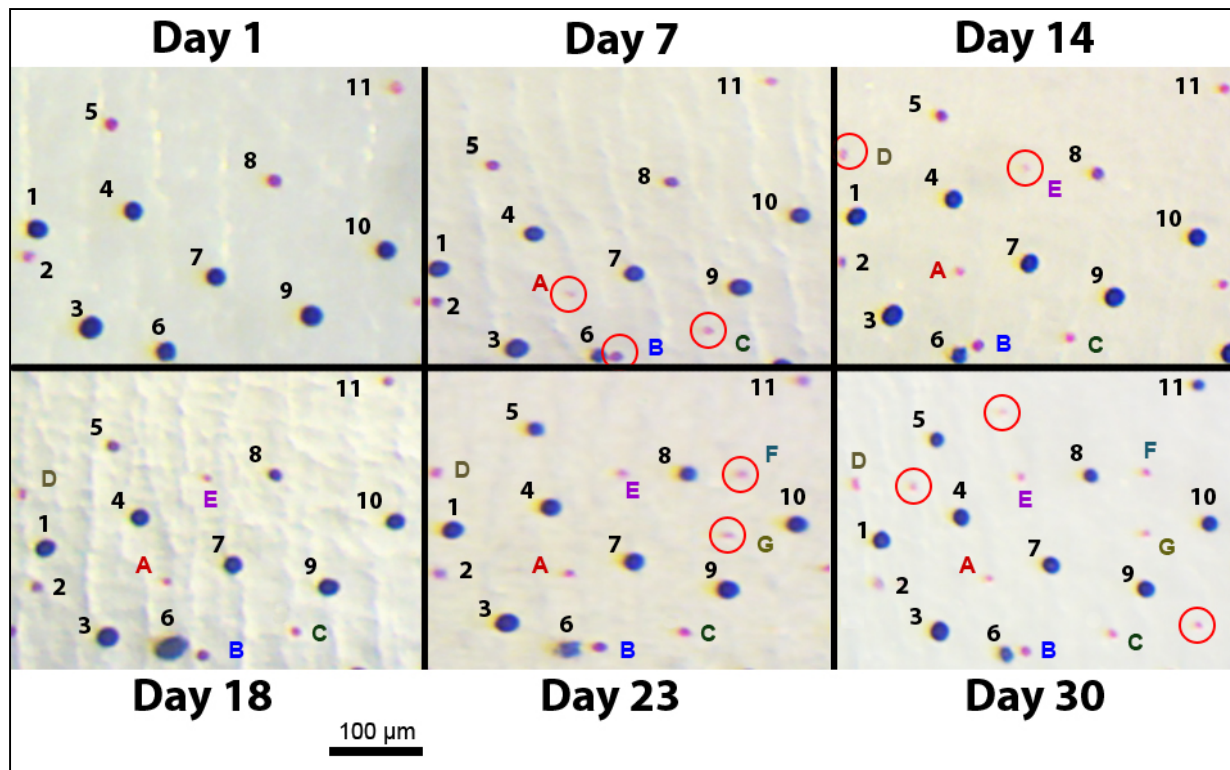
Figure 10. Time course of chromatophore appearance and maturation in the fin of a juvenile European cuttlefish *Sepia officinalis* **during normal growth.** This figure shows the addition of seven new chromatophores to the posterior fin of a juvenile *Sepia officinalis* during the 30 day observation period. Letters (A-G) show the location of new chromatophores and red circles indicate the day of their first appearance. Labels 1-11 show the location of existing chromatophores. Two existing chromatophores, #5 and #8, started out appearing as orange chromatophores and over time became darker until they were the same size and color as surrounding black chromatophores. Another chromatophore, #11, is observed partway through this maturation process.
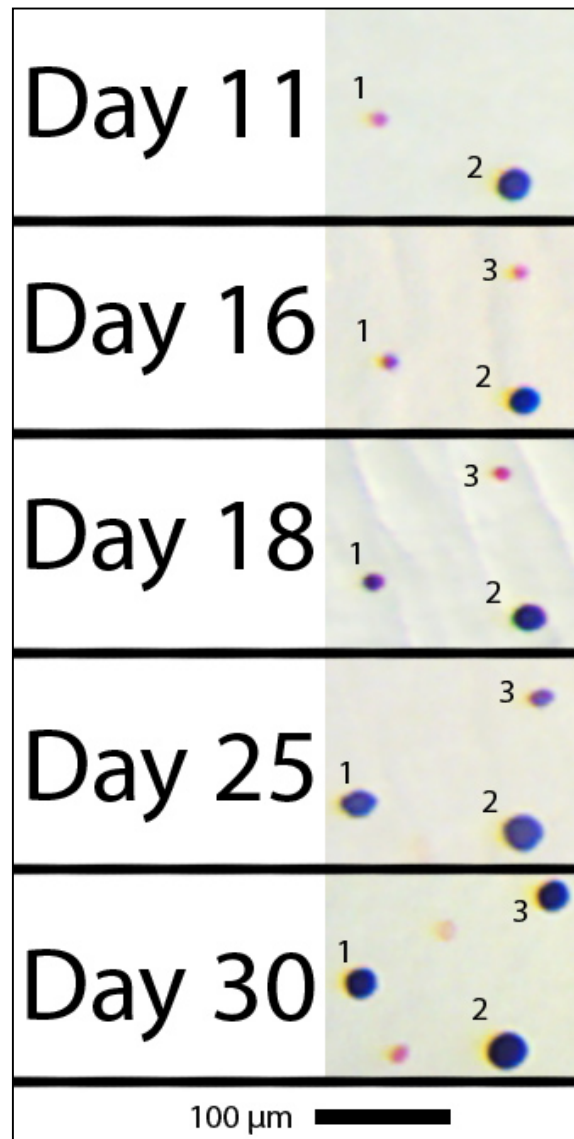
Figure 11. The ontogeny of black chromatophores during normal growth in the European cuttlefish *Sepia officinalis*. This figure shows the appearance and maturation of two black chromatophores (#1 & #3). Each new chromatophore starts as a pale orange color and slowly darkens and enlarges over the course of a few weeks until it is the same size and color as neighboring black chromatophores. At no stage does the chromatophore appear as fully grown yellow or reddish-brown chromatophore. Note the presence of a fully mature black chromatophore (#2) throughout the data collection period and of two new orange-colored chromatophores that appear on Day 25 and 30, respectively.
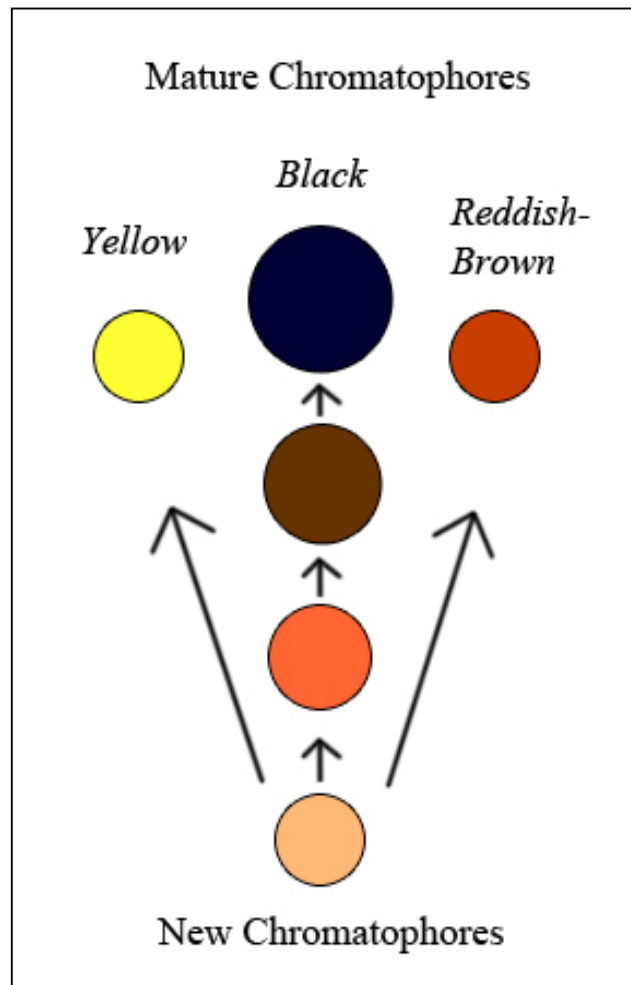
Figure 12. Schematic model of chromatophore maturation in the European cuttlefish *Sepia officinalis*.
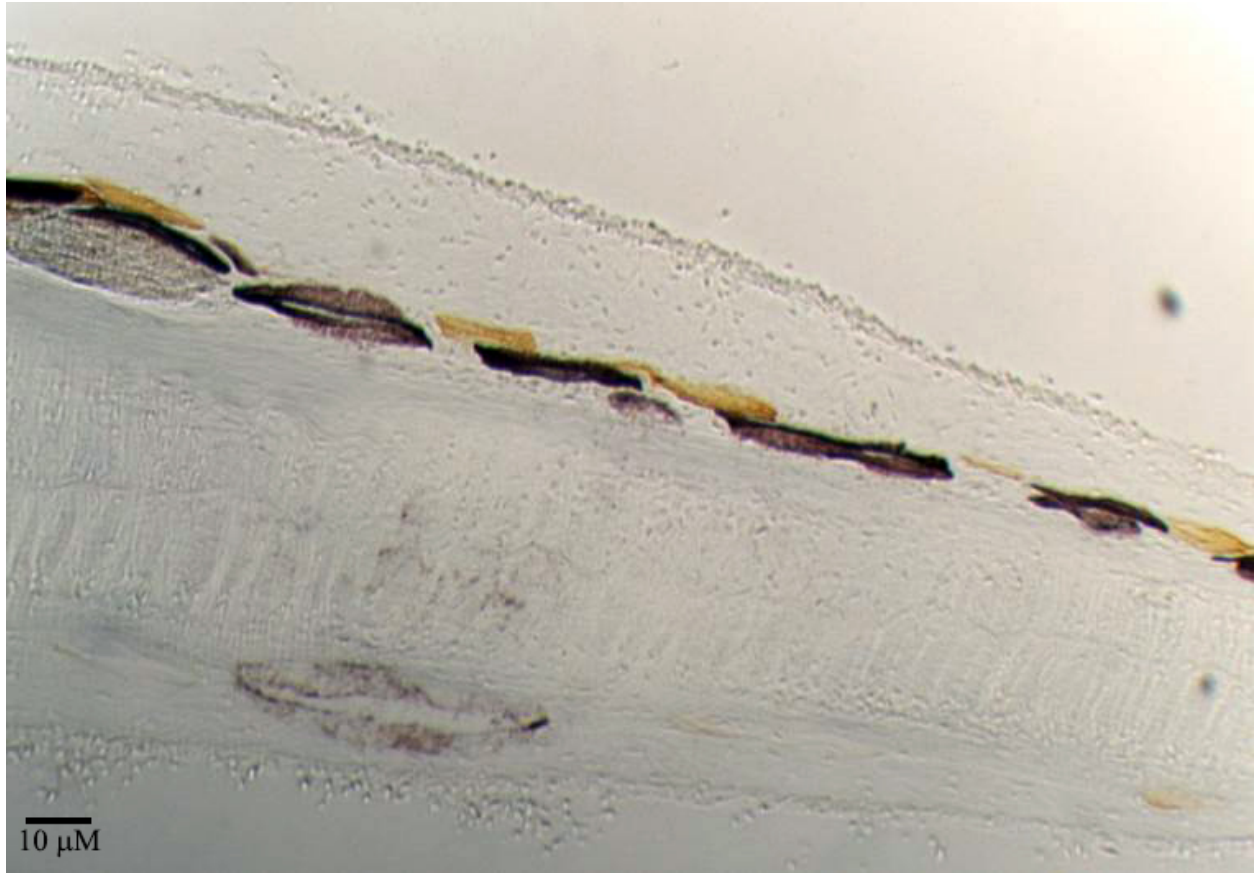
Figure 13. Unstained histological section of an adult cuttlefish fin showing only two layers of chromatophores, orange and black.
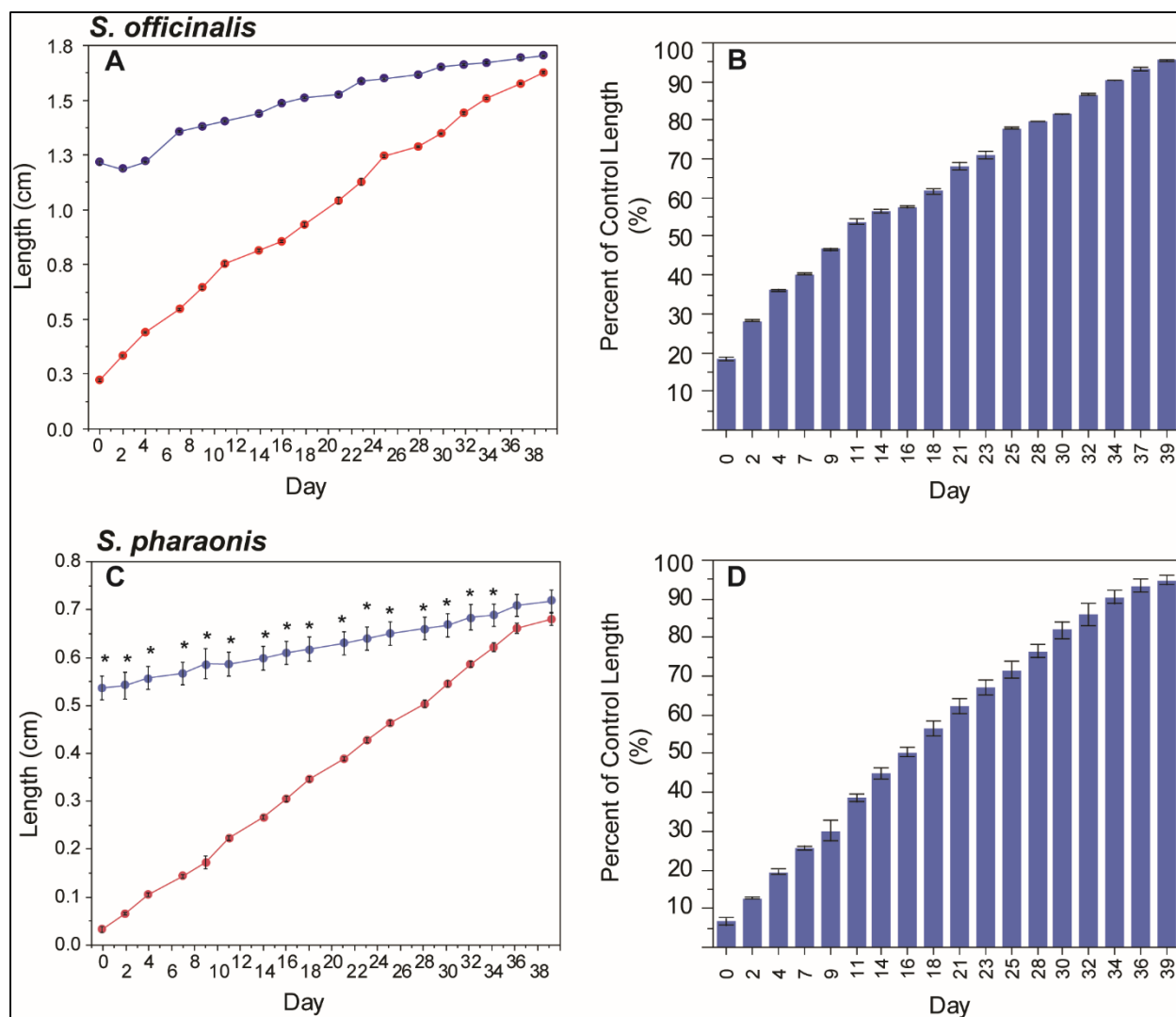
Figure 14. Arm Length of *Sepia officinalis* (top panels) and *Sepia pharaonis* (bottom panels) after amputation. **A & C:** Mean length of the control (blue) and regenerating (red) arm during the course of the experiment. Asterisks (*****) denote significant differences between the control and regenerating arm on a given day (α=0.05). **B & D:** Percent of regenerating arm length compared to control arm length ((length regenerating arm/length control arm) x 100). Each data point is expressed as a mean value ±1 standard error of the mean. The small size of error bars was due to a small amount of variability between individuals. N = 9 for *S. officinalis* and N = 4 for *S. pharaonis*.
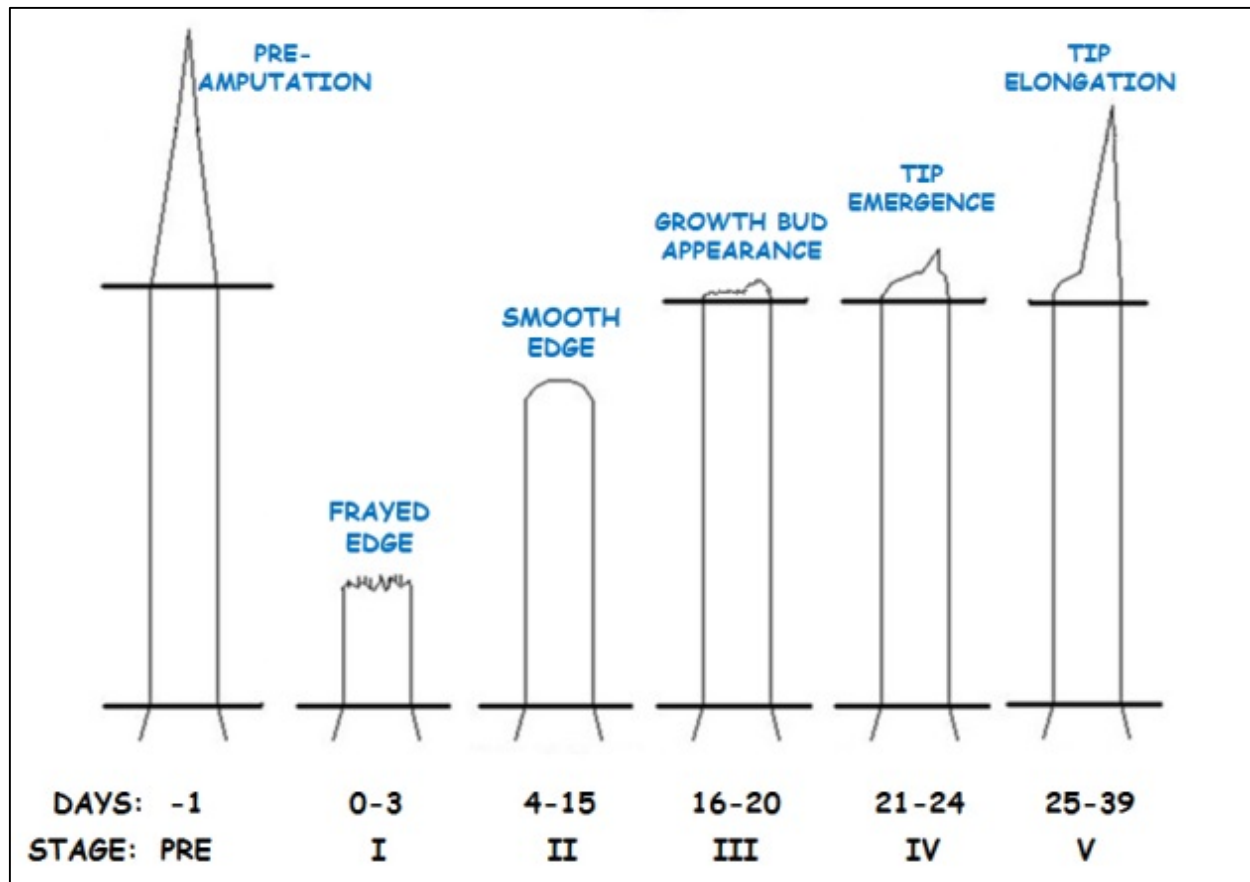
Figure 15. A summary of the stages of arm regeneration *in Sepia officinalis*. The bottom black line shows the site of amputation and the top black line shows the point when an intact arm begins to taper.

STAGE (*Sepia Officinalis*)	DAYS	NEW SUCTION CUPS	NEW C'PHORES	NORMAL SWIM-MING	NORMAL FOOD MANIPU-LATION	NORMAL BODY POSTURES	NORMAL BROWN TIP BEHAVIOR
I. Frayed Edge	0-3	YES	NO?	NO	NO	NO	NO
II. Smooth Edge	4-15	YES	YES	YES	NEARLY	NEARLY	NO
III. Growth Bud Appearance	16-20	YES	YES	YES	YES	YES	NO
IV. Tip Emergence	21-24	YES	YES	YES	YES	YES	NO
V. Tip Elongation	25-39	YES	YES	YES	YES	YES	YES

Figure 16. Behavioral and morphological responses to arm ablation in *Sepia officinalis*. The onset of each stage was determined by the initial appearance of the defining morphological characteristic.
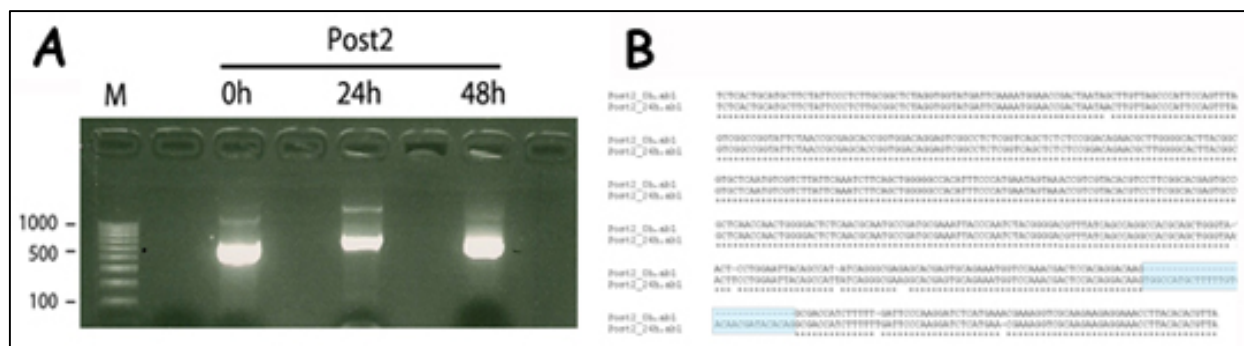

Figure 17. Expression and sequence of a regeneration-specific POST2 transcript in the regenerating arm of Sepia officinalis. A: Northern analysis of the POST2 transcript expression before (0 hr) and after (24 hr and 48 hr) arm amputation. Note the difference in size of the transcript at 24 hr and 48 hr compared to 0 hr. B: Sequences of the short (pre-amputation) and long (post-amputation) POST2 transcripts. The additional 60 bp of the long POST2 transcript is in blue.
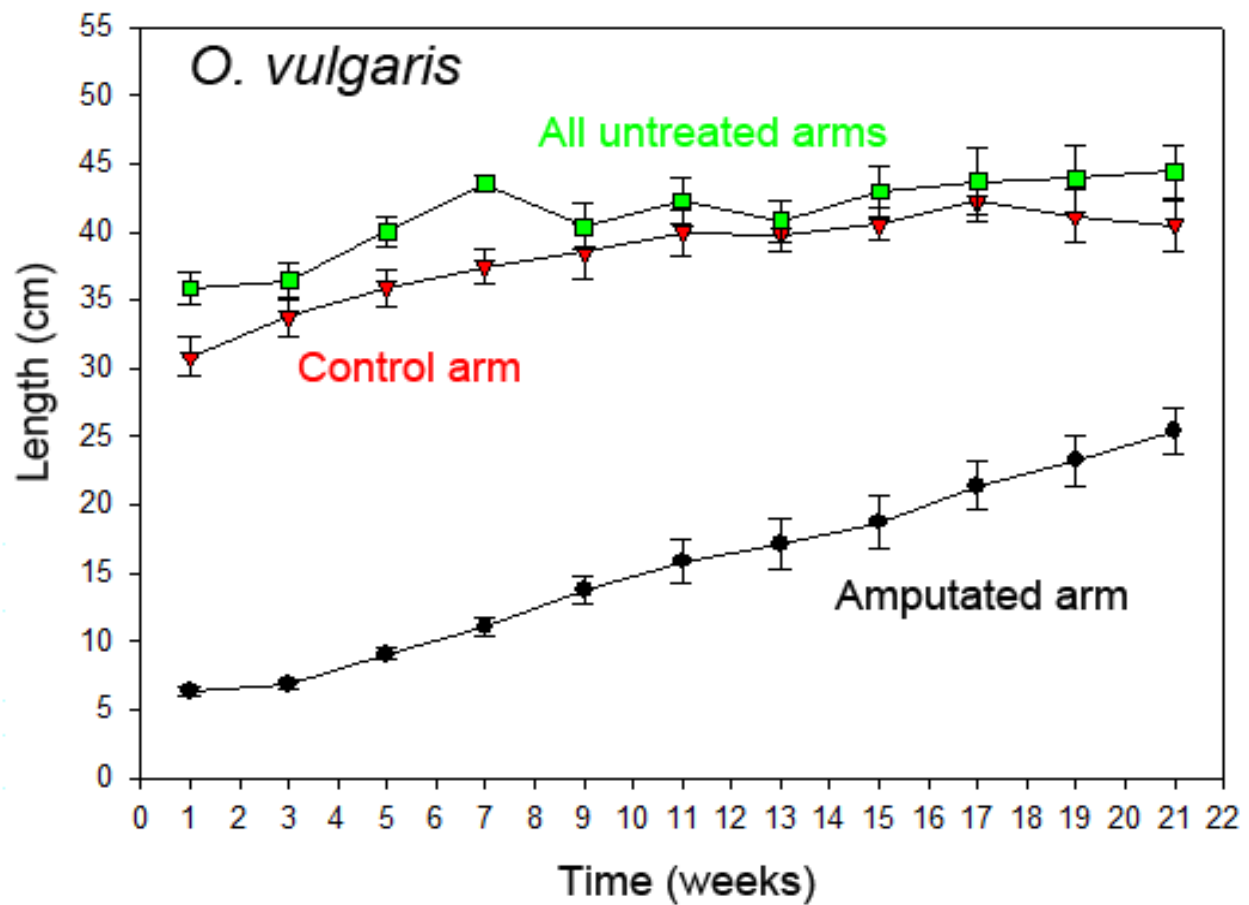
Figure 18. Time course of arm regeneration in the octopus *O. vulgaris*. Each animal had one arm severed, either the first right or first left arm. The untreated control arm was the same arm on the contralateral side. All untreated arms refers to all 7 untreated arms. Each point represents the mean +/- SEM of data obtained from 10 juvenile animals. Note that all untreated arms continued to grow throughout the duration of the experiment.
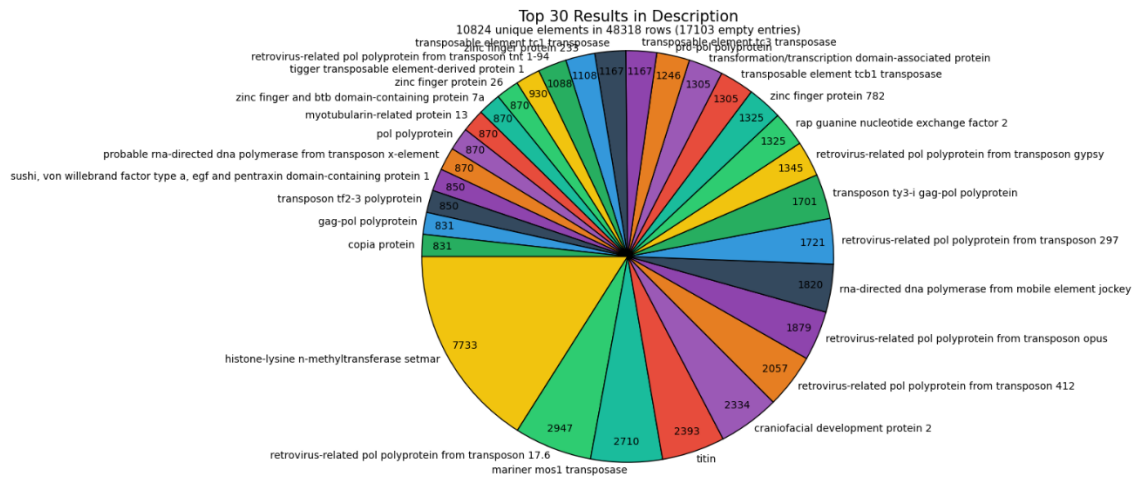
Figure 19. Transcriptional analyses of all 6 cuttlefish transcriptomes (see text for details). Top 30 matches to known proteins.
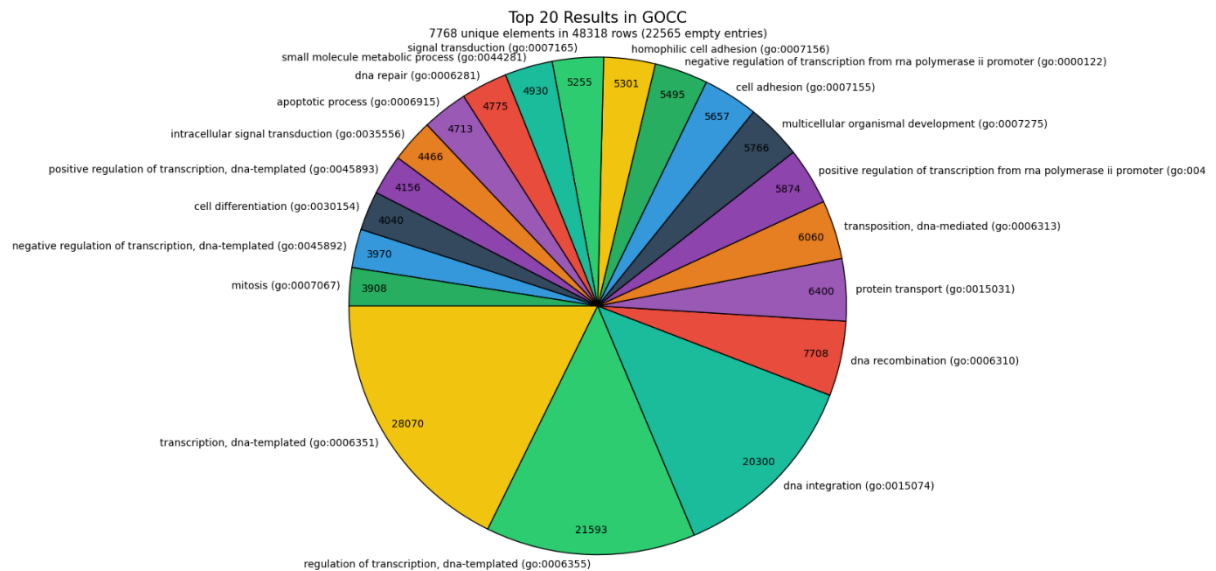

Figure 20. Transcriptional analyses of all 6 cuttlefish transcriptomes (see text for details). Top 20 matches to molecular functions.
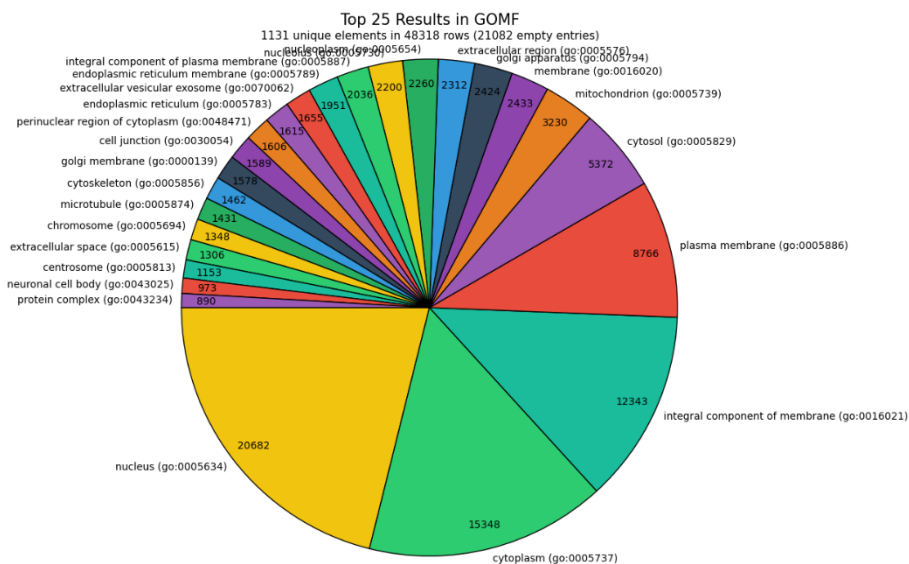
Figure 21. Transcriptional analyses of all 6 cuttlefish transcriptomes (see text for details). Top 25 matches to cellular compartment locations.
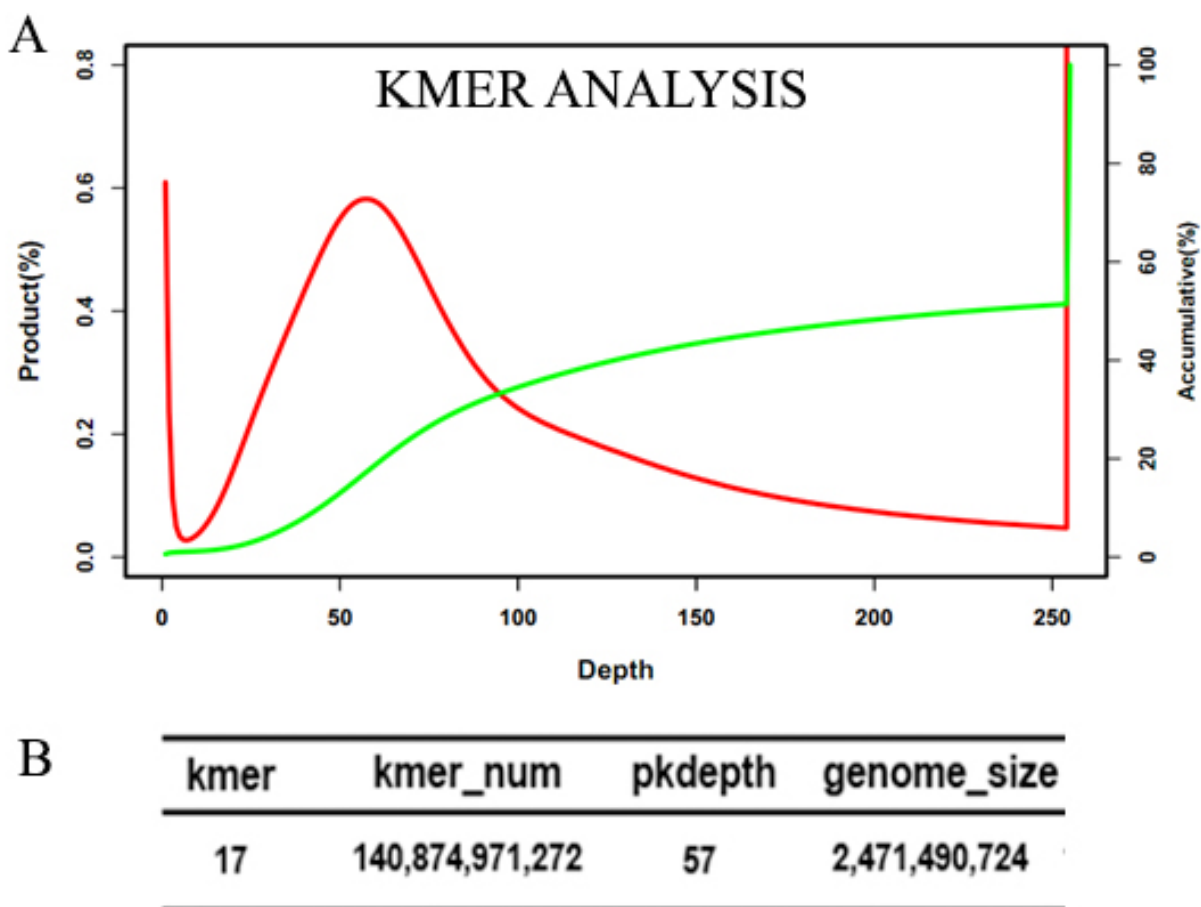
Figure 22. KMER analyses of the *Octopus vulgaris* genome. A.KMER results from 1 to 250 bases. B. Quantitative results from the maximum KMER peak depth of 57 bases. Genome size of ~2.5 Gb was determined by dividing kmer_num by pkdepth. Data obtained in collaboration with the Fiorito lab in Napoli, Italy.

Insert Size (bp)	Reads Length (bp)	Raw Data		Clean Data	
		Total Data(G)	Sequence coverage(X)	Total Data(G)	Sequence coverage(X)
170	100_100	89.14	17.83	77.24	15.45
250	150_150	56.47	11.29	47.75	9.55
500	100_100	70.22	14.04	58.14	11.63
800	100_100	61.72	12.34	49.87	9.97
Total	--	277.56	55.51	232.99	46.60

Figure 23. Alternative analyses of genome size of *O. vulgaris*. Estimated genome size of ~5 Gb was determined by dividing Total Data (G) / Total Sequence coverage (X). Data obtained in collaboration with the Fiorito lab in Napoli, Italy.
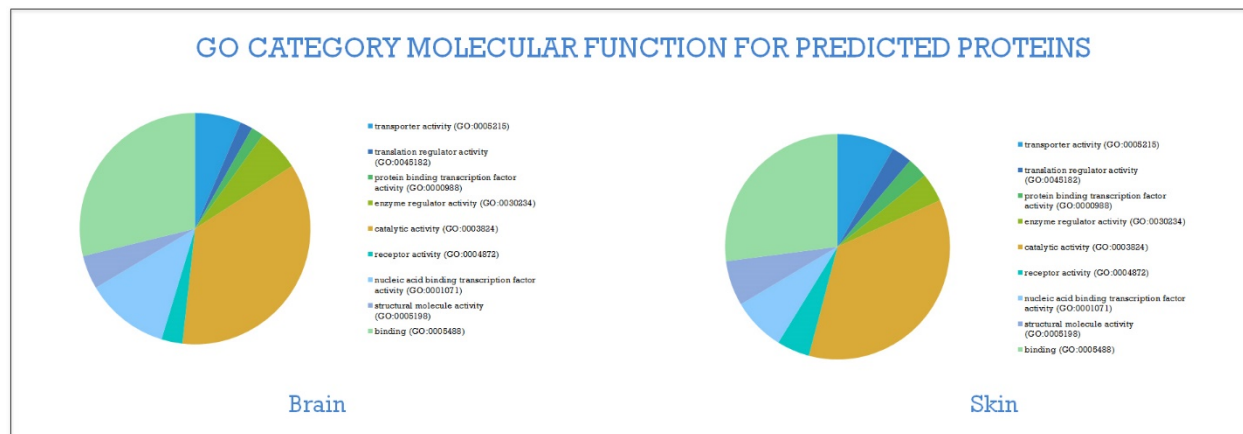

Figure 24. Initial characterization of annotated proteins from the skin and brain of the European cuttlefish *Sepia officinalis* based on genomic sequence data. Data were obtained in collaboration with the Human Genome Sequencing Center at the Baylor College of Medicine.

1.

1. Report Type

Final Report

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Organization / Institution name

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The full title of the funded effort.

NEURAL REGULATION OF CHROMATOPHORE FUNCTION IN CEPHALOPODS

Grant/Contract Number

AFOSR assigned control number. It must begin with "FA9550" or "F49620" or "FA2386".

FA9550-09-1-0395

Principal Investigator Name

The full name of the principal investigator on the grant or contract.

NATHAN TUBLITZ

Program Manager

The AFOSR Program Manager currently assigned to the award

Dr. HUGH DELONG

Reporting Period Start Date

06/01/2009

Reporting Period End Date

02/28/2015

Abstract

The long term goal of this research project was to elucidate some of the neural mechanisms underlying Body Pattering Behavior, the ability of cephalopod mollusks to generate numerous and highly complex body patterns. Cephalopods, which include octopus, squid and cuttlefish, are the only animals able to generate active body patterns directly controlled by the nervous system. These animals form numerous static and dynamic patterns used for a variety of purposes, including camouflage and inter- and intra-specific communication. The patterns made by these animals are unique because of their rich detail, speed of formation (within 500 ms), and high degree of plasticity. We have been studying Body Patterning Behavior in the European cuttlefish Sepia officinalis, which arguably generates the most detailed and varied body coloration patterns in the animal kingdom. The focus of this research is to understand the neural control of the color producing elements, the chromatophores. The specific objectives of this project address several key issues of chromatophore regulation.

OBJECTIVES

The four specific objectives/aims of this project were:

1. Identify the neurotransmitters controlling yellow and reddish-orange chromatophores;
2. Clarify the role of the FMRFamide-related peptides (FaRPs) in chromatophore function;
3. Characterize the central location and properties of the chromatophore motoneurons; and,
4. Determine the rules underlying addition of new chromatophores during maturation and growth.

SUMMARY OF FINDINGS:

Specific Aim #1: We determined that the yellow and reddish-orange chromatophores are controlled by three transmitters, glutamate, the FaRPs, and serotonin. Glutamate and the FaRPs serve as the fast and slow excitatory transmitters, respectively, in this system whereas serotonin acts as the inhibitory transmitter.

Specific Aim #2: We isolated, cloned and sequenced two new FaRP genes in cuttlefish that code for 8 novel neuropeptides, determined their expression pattern in the cuttlefish CNS, and demonstrated that these peptides each act to cause a slow excitation of the chromatophore muscles.

Specific Aim #3: We identified the location and properties of chromatophore motoneurons that activate chromatophores covering leucophores.

Specific Aim #4: We ascertained the rules by which new chromatophores are added to the existing chromatophore array during maturation and growth.

In addition,

1) We determined that there are only two color types of chromatophores in cuttlefish, not three as previously assumed.

2) We quantitatively assessed the rate of regeneration of a severed arm in two species of cuttlefish.

3) We identified, cloned and sequenced a specific Hox gene, POST2, which has an alternative transcript only expressed in regenerating arm tissue.

4) We quantitatively measured the rate of regeneration of a severed arm in a related cephalopod species, Octopus vulgaris with the Fiorito lab at the Stazione Zoologica in Napoli, Italy and showed that regeneration follows a different time course than that in cuttlefish.

5) We performed a transcriptomal analysis on 6 different cuttlefish tissues, including the brain, retina, chromatophore layer, normal arm and arms 24 and 48 hrs after transection.

6) We generated an Octopus vulgaris genome survey to determine the size of the Octopus genome in collaboration with the Fiorito lab at the Stazione Zoologica in Napoli, Italy.

7) We began a large scale effort in collaboration with the Human Genome Sequencing Center at the Baylor College of Medicine to fully sequence, assemble and annotate the cuttlefish genome, which is 50% larger than the human genome. To date we have completed the sequence and are in the process of assembly and annotation.

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Archival Publications (published) during reporting period:

Yacob, J., A.C. Lewis, A. Gosling, D. H.J.St Hilaire, L. Tesar, M. McRae and N.J. Tublitz (2011) Principles underlying addition of new chromatophores in the European cuttlefish Sepia officinalis. J. Exp. Biol., 214: 3423-3432.

Zhang, Z., E. Goodwin, P.K. Loi and N.J. Tublitz (2012) Molecular analysis of a novel FMRFamide-related peptide gene (SOFaRP2) and its expression pattern in the brain of the European cuttlefish Sepia officinalis. Peptides 34: 114-119.

Goodwin, E., and N.J. Tublitz (2013) Video analyses of chromatophore activity in the European cuttlefish Sepia officinalis. J. Exp. Marine Biol. & Ecol., 447:156-159.

Zhang, Z, and N.J. Tublitz (2013) Expression of the SOFaRP2 gene in the central nervous system of the European cuttlefish Sepia officinalis. Neuropeptides 47: 147-155.

Tressler, J., F. Maddox, E. Goodwin, Z. Zhang and N.J. Tublitz (2014) Arm regeneration in two species of cuttlefish, Sepia officinalis and Sepia pharaonis. Invert. Neurosci., 14: 37-49. DOI 10.1007/s10158-013-0159-8.

Fiorito, G., A. Affuso, D. B. Anderson, J. Basil, L. Bonnaud, G. Botta, A. Cole, L. D'Angelo, P. De Girolamo, N. Dennison, L. Dickel, A. Di Cosmo, C. Di Cristo, C. Gestal, R. Fonseca, F. Grasso, T. Kristiansen, M. Kuba, F. Maffucci1, A. Manciocco, F. C. Mark, D. Melillo, D. Osorio, A. Palumbo, K. Perkins, G. Ponte, M. Raspa, N. Shashar, J. Smith, D. Smith, A. Sykes, R. V. López, N. Tublitz, L. Zullo, and P. Andrews (2014). Cephalopods in Neuroscience: Regulations, Research and the 3Rs. Invert. Neurosci., 14: 13-36. DOI 10.1007/s10158-013-0165-x

Zhang, Z. and N.J. Tublitz (2015) Identification and expression of a regeneration-specific, splice variant of the POST Hox gene in arm regeneration in the European cuttlefish Sepia officinalis. In preparation.

Zarrella, I., G. Fiorito and N J. Tublitz (2015) Morphometric and behavioral analyses of arm regeneration in the common octopus Octopus vulgaris. In preparation.

Changes in research objectives (if any):

N/A

Change in AFOSR Program Manager, if any:

N/A

Extensions granted or milestones slipped, if any:

2 extensions granted.

AFOSR LRIR Number

LRIR Title

Reporting Period

Laboratory Task Manager

Program Officer

Research Objectives

Technical Summary

Funding Summary by Cost Category (by FY, $K)

	Starting FY	FY+1	FY+2
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Equipment/Facilities			
Supplies			
Total			

Report Document

Report Document - Text Analysis

Report Document - Text Analysis

Appendix Documents

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